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Impairment of Na^+/H^+ exchange underlies inhibitory effects of Na^+-free media on leukocyte function

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Inhibition of activation has been reported when neutrophils are suspended in Na^+-free media. We considered the possibility that impairment of cellular pH (pH_i) regulation due to elimination of Na^+/H^+ exchange underlies this effect. In the absence of Na^+, the phorbol ester-induced respiratory burst was partially inhibited and a concomitant cytoplasmic acidification recorded. Using nigericin/K^+ to clamp pH_i we demonstrated that the acidification accounts for the inhibition of O_2 uptake. Moreover, in Na^+-free media, relieving the acidification by means of ionophores restored maximal O_2 consumption. It was concluded that Na^+ is not directly involved in signal transduction during stimulation. Instead, omission of Na^+ affects neutrophile activation indirectly, by impairing pH_i regulation.

pH regulation *Phorbol ester* *Oxygen consumption* *Superoxide* *Neutrophil*

1. INTRODUCTION

When activated, neutrophils undergo a number of morphological and metabolic changes. These responses, which serve to enhance the microbial killing capacity of the neutrophils, include the activation of phagocytosis and chemotaxis, stimulation of the hexose monophosphate shunt, degranulation, and an increased uptake of O_2 [1]. The O_2 consumption during the metabolic burst is cyanide-insensitive and appears to be largely for the production of superoxide anion (O_2^-), as well as hydrogen peroxide and other oxygen radicals [2].

Activation is also accompanied by an amiloride-sensitive influx of Na^+, which has been attributed to activation of the Na^+/H^+ exchanger (antiport) [3–6]. The Na^+/H^+ exchanger, which is virtually quiescent in resting (unstimulated) neutrophils, can be activated by acidification of the cytoplasm, suggesting a role in the regulation of intracellular pH (pH_i) [7]. It has been reported that when the antiport is inhibited either by omission of extracelllar Na^+ or by addition of amiloride, the activation of neutrophils by chemotactic factors or by phorbol esters is accompanied by a marked cytoplasmic acidification [6,8]. This large acidification, which is not detectable when external Na^+ is present, has been attributed to increased acid production during the metabolic burst [8,9].

It has been reported that substitution of extracellular Na^+ by K^+ or by organic monovalent cations results in partial inhibition of the responses of activated neutrophils, including the generation of superoxide [10–12]. Because the pH optimum of many enzymes is relatively narrow, we hypothesized that inhibition of the responses of neutrophils activated in Na^+-free media might actually arise from changes in pH_i, rather than from direct effects of the alkali cation on ligand binding, signalling or activation. Instead, the effects would be secondary to inhibition of Na^+/H^+ exchange and the ensuing cytoplasmic acidification. The aim of the present experiments was to establish whether the respiratory burst was affected by the removal of Na^+ from the medium, and to determine if these effects are attributable to alterations of pH_i. For

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this purpose, we measured O_2 consumption in human blood neutrophils activated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). This lipid-soluble agent readily traverses the plasma membrane and directly activates protein kinase C, thereby bypassing any possible effects of external cation substitution on the binding of impermeant activators to their cell surface receptors [13].

2. MATERIALS AND METHODS

2.1. *Reagents*

10-fold concentrated medium RPMI 1640 (HCO_3^- free) was purchased from Gibco (Grand Island, NY). Hepes, *N*-methyl-D-glucamine (NMG), 2-(*N*-morpholino)ethanesulfonic acid (Mes), Tris and TPA were purchased from Sigma (St. Louis, MO). Nigericin was from Calbiochem-Behring (San Diego, CA). Ficoll 400 and dextran T500 were from Pharmacia (Uppsala). 2,7-Biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester was from Molecular Probes (Junction City, OR).

2.2. *Solutions*

Hepes-RPMI was prepared by addition of 20 mM Hepes to HCO_3^--free RPMI 1640 and titration with NaOH to pH 7.3. Na^+ solution contained (in mM) 140 NaCl, 5 KCl, 10 glucose, and 15 Tris-Mes. K^+ solution and NMG^+ solution contained 140 KCl and 140 NMG-Cl, respectively, instead of NaCl. The pH of these media was adjusted at 37°C to the values indicated in the text. Osmolarity was adjusted to 290 ± 5 mosM with the major salt. Stock solutions of BCECF acetoxymethyl ester (1 mg/ml) and TPA (10^{-5} M) in dimethyl sulfoxide and of nigericin (1 mM) in ethanol were stored at −20°C for several months.

2.3. *Cell isolation*

Neutrophils were isolated from freshly heparinized blood from healthy human donors by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation [14]. Contaminating red blood cells were then removed by ammonium chloride lysis. The cells were washed, resuspended in Hepes-RPMI at 10^7 cells/ml, and maintained at room temperature for up to 5 h. Immediately before use, the cells were sedimented in an Eppendorf microfuge and resuspended in the indicated medium at 2×10^6/ml.

2.4. *Cytoplasmic pH determination and manipulation*

Cytoplasmic pH was determined fluorimetrically. Cell suspensions (10^7 cells/ml in Hepes-RPMI) were loaded with the probe BCECF by incubation with the parent acetoxymethyl ester (1 μg/ml) for 30 min at 37°C. The cells were then washed and resuspended in Hepes-RPMI. Prior to each determination, 2×10^6 cells were sedimented and resuspended in 1 ml of the indicated medium. Fluorescence was measured at 37°C with stirring using a Perkin-Elmer 650-40 fluorimeter, with excitation at 485 nm and emission at 540 nm using 5 and 10 nm slits, respectively. The K^+/nigericin method of Thomas et al. [15] was used for calibration and for clamping of pH_i (see section 3).

2.5. *O_2 consumption*

O_2 consumption was measured with a model 53 biological oxygen monitor (Yellow Springs Instruments), which utilizes a Clark type polarographic electrode. Cells (4×10^6) were suspended in 2 ml of the indicated medium at 37°C and stirred magnetically. O_2 uptake was monitored continuously using a Y vs time chart recorder. The O_2 electrode was titrated with dithionite in the various media to ensure that its performance was not affected by pH. O_2 consumption was calculated using a solubility coefficient of 0.024 ml O_2/ml medium at 37°C.

All measurements were made at least 3 times using different donors. Unless otherwise indicated, the data are presented as means ± SE of the number of determinations indicated.

3. RESULTS AND DISCUSSION

The rate of O_2 consumption in the 0–2 min interval in TPA-treated neutrophils was somewhat faster in Na^+-containing than in Na^+-free (K^+ or NMG^+) media (fig.1A and table 1). The rate of O_2 consumption decreased with time in both types of media, but the decrease was more pronounced in Na^+-free media. After 4–6 min the rate was 5.1 nmol $O_2 \cdot 10^6$ cells$^{-1} \cdot$ min^{-1} in Na^+ solution but only 4.0 nmol $O_2 \cdot 10^6$ cells$^{-1} \cdot$ min^{-1} in NMG^+ solution and 4.1 nmol $O_2 \cdot 10^6$ cells$^{-1} \cdot$ min^{-1} in K^+

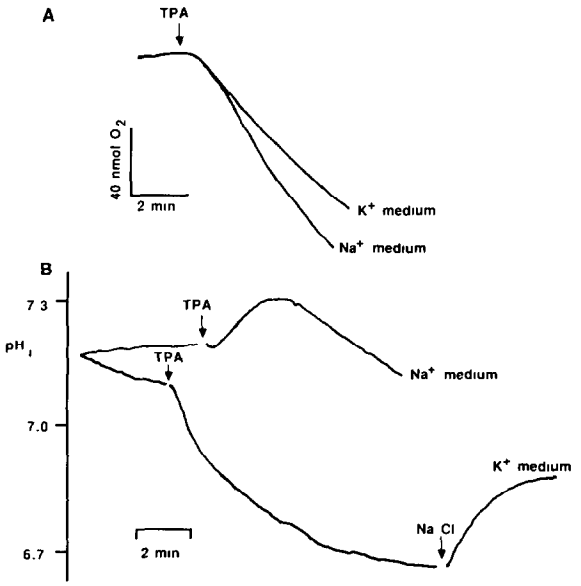

Fig.1. O_2 uptake and pH_i of neutrophils following activation with TPA in Na^+ and K^+ media. Tracings were obtained as outlined in section 2 and are representative of at least 3 experiments. A 10^{-5} M stock of TPA in DMSO was added where indicated by arrow to a final concentration of 10^{-8} M. (A) O_2 consumption measured with a Clark electrode. 2×10^6 cells/ml were suspended in the indicated medium (pH 7.3) 3.5 min before the start of the trace. (B) 2×10^6 BCECF-loaded cells were suspended in 1 ml of the indicated medium at the start of the trace. Breaks in the traces indicate opening of the sample compartment for additions. Where indicated, 30 μl of 2 M NaCl was added to the sample in K^+ solution for a final concentration of 60 mM. Temperature: 37°C.

solution (table 1). To determine if these differences could be related to changes in pH_i, this parameter was measured fluorimetrically under comparable conditions (fig.1B). Consistent with previously reported results [8], activation in Na^+ medium produced an incipient acidification followed by a small and variable alkalinization. The alkalinization was transient, pH_i showing a maximum increase of about 0.15 pH units 4 min after activation and then decreasing. In K^+ medium (fig.1B) and in NMG^+ medium (fig.4D), activation caused a rapid acidification of the cytoplasm. Cytoplasmic acidification was also seen following activation in Na^+ medium containing 300 μM amiloride (not shown), indicating that the acidification is not due to reversal of the Na^+/H^+ antiport. Instead, it is likely due to the accumulation of metabolic acid produced by stimulation of the hexose monophosphate shunt and/or the NADPH oxidase [8,9]. Further evidence that the cytoplasmic acidification arose from impairment

Table 1

Effects of external cation substitution on O_2 consumption and on pH_i

Medium	O_2 consumption (nmol $O_2 \cdot 10^6$ cells$^{-1} \cdot$ min^{-1})			pH_i		
	Initial	4–6 min	+ Nig	Initial	4 min	+ Nig
Na^+	5.6 ± 0.3	5.1 ± 0.3	4.1 ± 0.1	7.10 ± 0.02	7.26 ± 0.03	[a]
NMG^+	5.0 ± 0.6	4.0 ± 0.3	0.9 ± 0.0	7.00 ± 0.05	6.65 ± 0.02	6.17 ± 0.03
K^+	4.8 ± 0.2	4.1 ± 0.2	6.1 ± 0.5	6.93 ± 0.04	6.58 ± 0.02	7.12 ± 0.02

[a] See p. 84

O_2 consumption: 4×10^6 cells were suspended in 2 ml of the specified media approximately 4 min before activation. O_2 consumption was measured as described in section 2. The initial rate represents the maximum slope, which was achieved within 2 min of activation. The second column represents the slope between 4 and 6 min after activation. Third column: rate attained after addition of 5 μM nigericin. pH_i: BCECF-loaded cells were suspended at 2×10^6/ml in the indicated media approx. 4 min before activation. The initial value represents pH_i immediately before TPA activation; the pH_i attained 4 min after the addition of TPA is listed in the second column; the pH_i achieved within 2 min after the addition of nigericin is given in the third column. The data are means ± SE of 3 experiments

of antiport activity by omission of Na^+ is presented in fig.1B. Addition of 60 mM external Na^+ (arrow in fig.1B) to cells allowed to acidify in K^+ solution induced an immediate alkalinization.

In view of the results presented in fig.1B, it was conceivable that the differences in the rate of O_2 consumption in the presence and absence of external Na^+ were due to differences in pH_i resulting from the presence or absence of Na^+/H^+ exchange. This possibility was analyzed further by measuring the rate of O_2 consumption as a function of pH_i. For these experiments, unlike those in fig.1, pH_i was set at a desired initial value and maintained virtually constant throughout the course of the measurement. This was accomplished by suspending the cells in K^+ solution in the presence of the K^+/H^+ ionophore nigericin. This procedure is based on the premise that, in the presence of high nigericin concentrations, pH_i will reach a steady state when $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$. Because the intracellular concentration of K^+ is similar to that in K^+ solution, pH_i will approximate pH_o. Thus, pH_i clamping was obtained by suspending cells in K^+ media of varying pH_o and adding 5 μM nigericin.

A typical experiment validating the pH_i clamping technique is shown in fig.2. The initial pH_i of the cells was 7.06 ± 0.01. When suspended in K^+

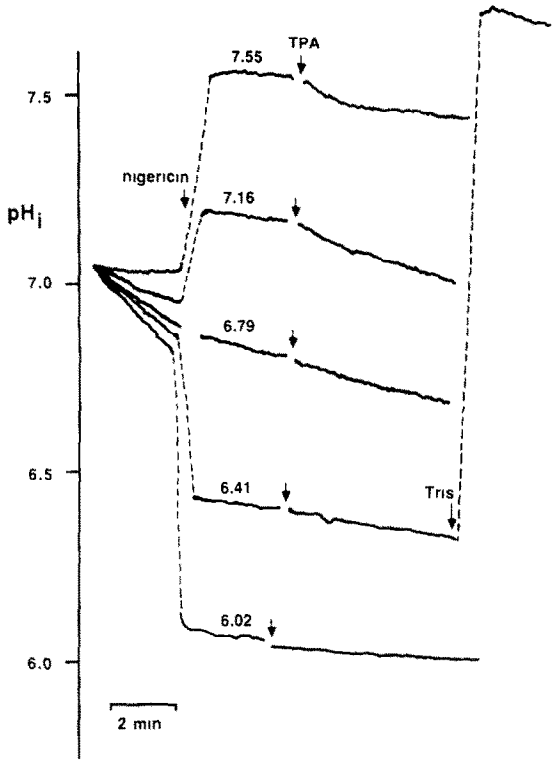

Fig.2. Manipulation of pH_i using K^+/nigericin. 2×10^6 BCECF-loaded cells were suspended in 1 ml of K^+ solution of the indicated pH at the start of the trace. Where indicated, nigericin was added to a final concentration of 5 μM. After pH_i had reached equilibrium, a combination electrode was inserted into the cuvette and the pH recorded. TPA (10^{-8} M, final) was added where indicated. 5 μl of 1 M Tris base was added to one of the samples where indicated to raise pH_i to 7.60. The figure is a composite of several traces obtained from the same preparation. The composite is representative of 4 similar experiments.

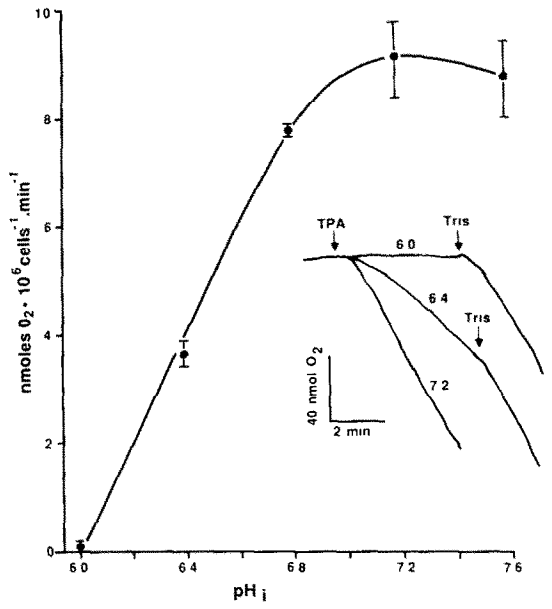

Fig.3. Effect of pH_i on O_2 consumption. (Inset) Consumption of O_2 in K^+ media of varying pH containing nigericin. Cells (2×10^6/ml) were suspended in K^+ medium of the indicated pH 4.5 min prior to the start of the trace. Nigericin was added 1 min prior to the start of the trace. 10 μl of 1 M Tris was added to the pH 6.4 and 6.0 samples where indicated to raise pH to 7.66 and 7.25, respectively. Representative of 4 determinations in samples from 3 different donors. (Main panel) Rate of O_2 consumption as a function of pH_i. Experiments were performed as above. Rate was measured as the maximum slope, which was achieved within 2 min of the addition of TPA. Points represent the mean ± SE of 3 experiments.

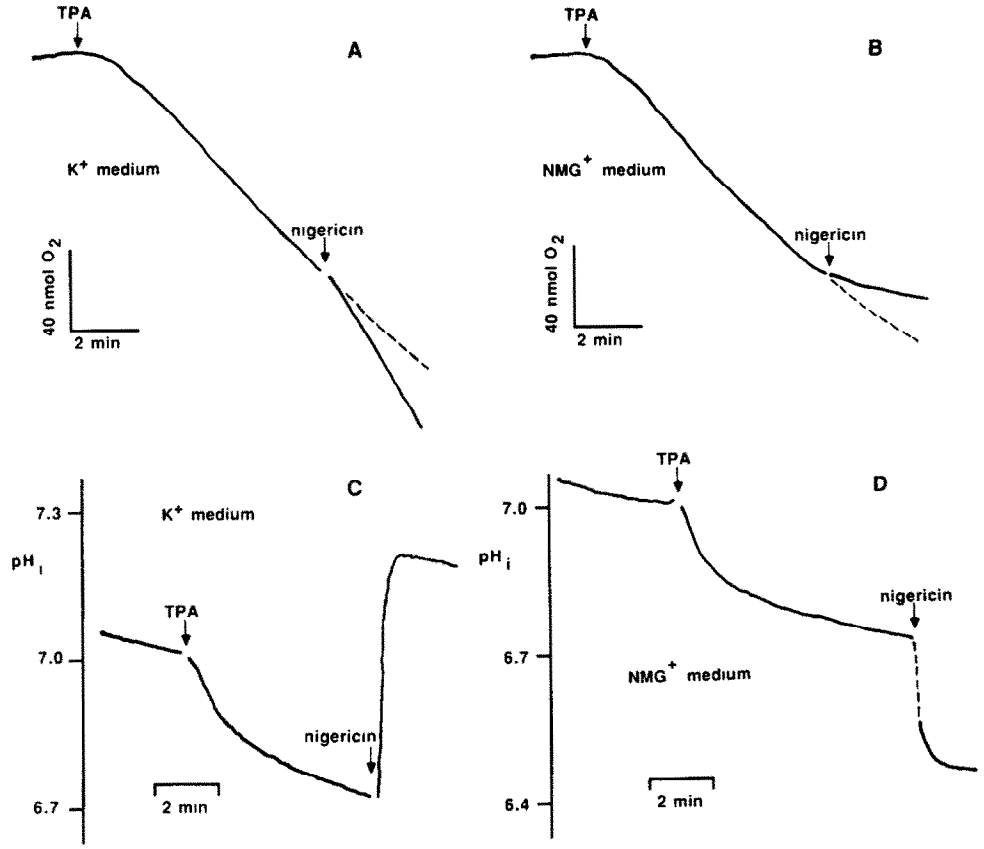

Fig.4. Effects of nigericin on O_2 consumption and pH_i of cells in K^+ and *N*-methyl-D-glucamine$^+$ (NMG^+) media. (A,B) O_2 consumption. Cells were suspended in either K^+ medium (A) or NMG^+ medium, pH 7.2 (B) 4 min prior to the start of the trace. 10^{-8} M TPA and 5 μM nigericin were added where indicated by arrows. The dashed line indicates the extrapolated rate in the absence of nigericin. (C,D) Cytoplasmic pH (pH_i). Cells were suspended in K^+ (C) or NMG^+ (D) medium at the start of the trace. Traces are representative of 3 similar experiments.

media of pH_o 7.2 or lower, the cells acidified spontaneously, possibly due to leakage of extracellular H^+ equivalents, to the accumulation of metabolic acid and/or to the reverse operation of the Na^+/H^+ exchanger. Addition of nigericin caused a rapid equilibration of pH_i with pH_o. Under these conditions, the pH_i was relatively stable, showing minimal drift. When concentrated acid or base was added to the medium, pH_i quickly equilibrated with the new pH_o (e.g. addition of Tris base in fig.2). Activation of the cells with TPA caused only slight (<0.1 unit) changes in pH_i (cf. fig.1B), since the H^+ equivalents generated by the respiratory burst were rapidly shunted out of the cells by the ionophore.

The effect of varying pH_i on TPA-induced O_2 consumption is shown in fig.3. A typical experiment is illustrated in the inset, where the cells were treated with nigericin in K^+ media of the indicated pH prior to the addition of the phorbol ester. The rate of TPA-induced O_2 consumption was maximal at pH 7.2 and decreased sharply at more acidic levels (60% inhibition at pH 6.4 and 99% inhibition at 6.0). The inhibition was reversible even under conditions where O_2 consumption was completely eliminated, since readjustment of the extracellular pH restored O_2 consumption to near maximal levels (inset, fig.3). Data from 3 similar experiments are summarized in the main graph of fig.3. O_2 consumption averaged 9.0 ± 0.7 nmol $O_2 \cdot 10^6$ cells$^{-1} \cdot$ min^{-1} at pH_i 7.2 [the maximal rates of O_2 consumption attained in K^+ solution, pH 7.2, in the presence of nigericin were significantly larger than the control rates, whether the

ionophore was added before (fig.3) or after (table 1) TPA. This may be due to a direct stimulatory effect of K^+ (or the associated depolarization) which is not manifested unless pH_i is maintained constant by nigericin], and it declined steadily as pH_i dropped from 6.8 to 6.0. Consumption was also slightly lower at pH 7.6, although the decrease was not significant. O_2 consumption by cells suspended in K^+ medium with nigericin in the absence of TPA was negligible.

Taken together, these results suggest that the effects of ion substitution on the respiratory burst arise from changes in pH_i. If the declining rate of O_2 consumption in K^+ medium shown in fig.1A is in fact due to cytoplasmic acidification (rather than to a direct effect of cation replacement), it should be possible to reverse the effect by restoring pH_i to physiological levels. As shown in fig.4C, this can be accomplished by adding nigericin after the phorbol ester has acidified the cells. The addition of the ionophore to TPA-activated cells suspended in K^+ medium at pH 7.2 caused a rapid alkalinization of pH_i. Concomitantly, the rate of O_2 consumption was markedly accelerated (fig.4A and table 1). That stimulation was due to the resulting changes in pH_i rather than the presence of nigericin itself could be demonstrated by adding the ionophore to cells suspended in NMG^+ solution (fig.4B,D). As expected for an Na^+-free medium, addition of TPA induced a marked acidification. A further acidification was observed when nigericin was added, since in the absence of extracellular K^+ the ionophore catalyzes the exchange of intracellular K^+ for extracellular H^+. Concomitant with the drop in pH_i, O_2 consumption declined from 4.0 to 0.9 nmol $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (fig.4B and table 1) [in Na^+ medium, the addition of nigericin caused a transient cytoplasmic acidification, probably due to K_i^+/H_o^+ exchange through the ionophore followed by Na_o^+/H_i^+ exchange through the antiport, which brought pH_i back to near normal levels within 4 min. O_2 consumption showed a small decrease, apparently due to the transient acidification (not shown)]. It is therefore the change in pH_i rather than the presence of nigericin itself that stimulates O_2 consumption in cells activated in K^+ medium.

Simchowitz [12] reported a positive correlation between the amount of O_2^- generated and the final pH_i value attained by fMLP-activated human neutrophils. He attributed the cellular acidification to the reverse operation of the Na^+/H^+ antiport. We have demonstrated that the acidification is also observed in the presence of Na^+ plus amiloride, ruling out this explanation. In addition, using the nigericin/K^+ technique to clamp pH_i prior to activation, we have shown a causal relationship between O_2 consumption and pH_i, and that the effects are largely reversible. Finally, because TPA was used in our studies, we can ascertain that acidification affects either protein kinase C or its target, rather than the receptor binding or transduction steps.

In summary, the present results demonstrate that in intact activated neutrophils the rate of O_2 consumption is sensitive to pH_i. Moreover, they confirm that, in the nominal absence of HCO_3^-, pH_i regulation is impaired when the cells are suspended in Na^+-free solutions. This may at least partially explain the decrease in superoxide generation and perhaps the decrease of other responses observed when neutrophils are suspended in media devoid of Na^+.

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